The EMBO Journal vol.14 no.18 pp.4412–4421, 1995

A new genetic selection identifies essential residues in SecG, a component of the *Escherichia coli* protein export machinery

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The signal sequence of the murine serine protease inhibitor PAI-2 promotes alkaline phosphatase export to the *E.coli* periplasm. However, high level expression of this chimeric protein interferes with cell growth. Since most suppressors of this toxic phenotype map to *secA* and *secY*, growth arrest results from a defective interaction of the chimeric protein with the export machinery. We have characterized suppressors which map in *secG*, a newly defined gene of the export machinery. All single amino acid substitutions map to three adjacent codons. These *secG* mutants have a weak Sec phenotype, as determined by their effect on export mediated by wild-type and mutant signal sequences. Whilst a *secG* disruption allele also confers a weak Sec phenotype, it does not suppress the toxicity of the chimeric protein. This difference results from a selective effect of the *secG* suppressors on the kinetics of export mediated by the PAI-2 signal sequence. Using a *malE* signal sequence mutant, which has a Mal$^-$ phenotype in *secG* mutant strains, we have isolated extragenic Mal$^+$ suppressors. Most suppressors map to *secY*, and several are allele-specific. Finally, SecG overexpression accelerates the kinetics of protein export, suggesting that there are two types of functional translocation complexes: with or without SecG.

Keywords: *E.coli*/PAI-2/*secG*/signal sequence/translocation

Introduction

The *Escherichia coli* protein export machinery has been extensively characterized both genetically (Schatz and Beckwith, 1990) and biochemically (Wickner *et al.*, 1991). Several genetic selections have been used to identify genes involved in preprotein translocation across the cytoplasmic membrane. Both recessive conditional lethal *sec* mutations resulting in a generalized secretion defect and dominant *prl* mutations affecting protein localization have been isolated. Early selections of *sec* mutants were based on the properties of strains expressing a hybrid protein containing the signal sequence of an exported protein fused to β-galactosidase, a cytoplasmic enzyme (Silhavy and Beckwith, 1985). These strains are Lac$^-$ due to the insertion of at least part of the fusion protein in the membrane. In addition, fusion of β-galactosidase to either maltose-binding protein (MBP) or LamB results in a maltose-sensitive phenotype caused by overproduction and jamming of the secretory apparatus (Ito *et al.*, 1981). A second approach involved the isolation of extragenic suppressors of signal sequence mutations (Emr *et al.*, 1981; Bankaitis and Bassford, 1985; Ryan and Bassford, 1985; Stader *et al.*, 1989). Finally, the observation that SecA expression is elevated in cells with a defect in protein secretion (Oliver and Beckwith, 1982) led to the isolation of additional *sec* mutants by selecting for the increased expression of a *secA–lacZ* fusion (Riggs *et al.*, 1988; Schatz *et al.*, 1989; Gardel *et al.*, 1990). These approaches led to the isolation of both *sec* and *prl* mutations in the *secA*, *secY* and *secE* genes, and only *sec* mutations in the *secB*, *secD* and *secF* genes.

The function of these Sec proteins has been extensively characterized biochemically. SecB is a cytoplasmic chaperone that maintains the precursor forms of several secreted proteins in a translocation competent state (Collier *et al.*, 1988; Kumamoto and Gannon, 1988; Altman *et al.*, 1991). SecA is a peripheral membrane ATPase (Lill *et al.*, 1989) which promotes preprotein translocation by undergoing 'ATP-driven cycles of membrane insertion/deinsertion' (Economou and Wickner, 1994). SecY and SecE form a membrane-embedded complex that supports preprotein translocation across the plasma membrane (Brundage *et al.*, 1992; Baba *et al.*, 1994). SecD and SecF are integral membrane proteins with large periplasmic domains which facilitate protein export (Pogliano and Beckwith, 1994); they are required for maintenance of a proton electrochemical gradient which, together with ATP, provides the energy necessary for protein translocation (Arkowitz and Wickner, 1994).

Protein translocation activity has been reconstituted into proteoliposomes with purified SecA, SecE and SecY (Brundage *et al.*, 1990; Akimaru *et al.*, 1991). The activity of reconstituted translocase was lower than that of intact inverted membrane vesicles (Akimaru *et al.*, 1991), but it could be greatly enhanced upon the addition of a membrane protein, P12 (Nishiyama *et al.*, 1993; Hanada *et al.*, 1994), whereas an antibody against P12 inhibited translocation into intact inverted membrane vesicles (Nishiyama *et al.*, 1993). P12 was recently shown to be identical to band1 (Douville *et al.*, 1994), a small protein which copurifies with the SecY–SecE complex (Brundage *et al.*, 1992). The P12-encoding gene maps at 69 min on the *E.coli* chromosome (Nishiyama *et al.*, 1993). Although the P12-encoding gene had never been identified in selections for *sec* or *prl* mutants, its disruption caused an accumulation of the precursors of exported proteins at 20°C and conferred a cold-sensitive phenotype. These observations led to the proposal that P12 may be an essential component of the translocation machinery, and its gene is now called *secG* (Nishiyama *et al.*, 1994).

This bacterial three-component translocase core appears similar in composition to the Sec61p complex of the mammalian and yeast ER translocation machinery. Whilst

Sec61pα and Sec61pγ are homologous to SecY and SecE at the amino acid sequence level (Görlich *et al.*, 1992; Hartmann *et al.*, 1994), Sec61pβ could be a SecG functional homologue despite the lack of sequence similarity. The conserved nature of the molecular organization of the translocase, the common requirement for structurally similar and interchangeable N-terminal signal sequences (von Heijne, 1985) and the existence in *E.coli* of an alternate SRP-like targeting pathway (Phillips and Silhavy, 1992; Luirink *et al.*, 1994; Miller *et al.*, 1994) have been proposed to support the existence of a conserved mechanism of protein export from bacteria to mammals.

In this study we describe a new selection for mutations in the genes encoding the components of the *E.coli* translocation machinery. We have characterized the export of PAI-2, a serine protease inhibitor which accumulates both in the cytosol and in the extracellular space of mammalian cells. This bitopological distribution of PAI-2 is mediated by an inefficient internal signal sequence, which is not cleaved (Ye *et al.*, 1988; Belin *et al.*,1989). We have fused the N-terminal region of PAI-2 to Tn*phoA*, which codes for a signal-less derivative of alkaline phosphatase that has been extensively used as a reporter for protein export to the periplasm (Manoil *et al.*, 1990). The N-terminal region of PAI-2 was recognized by the *E.coli* translocation machinery and promoted the export of alkaline phosphatase, albeit inefficiently. The chimeric protein was cloned in an expression vector containing the pBAD promoter of the arabinose operon (Guzman *et al.*, 1995). High level expression of the chimeric protein upon induction with arabinose led to irreversible growth arrest. This lethality is caused by a defective interaction of the chimeric protein with the export machinery. We have isolated extragenic suppressors that not only restored bacterial growth, but also allowed export of the chimeric protein. Whilst most of the suppressors map either to *secA* or *secY*, this new selection allowed us to isolate a mutation in *secG*. Extensive mutagenesis of this gene identified essential residues in the SecG component of the *E.coli* export machinery.

Results

Overexpression of a PAI-2–AP chimeric protein interferes with protein export

We have constructed a chimeric protein in which the N-terminal 57 residues of murine PAI-2 (mPAI-2; Belin *et al.*, 1989) are fused to the mature portion of *E.coli*'s alkaline phosphatase (AP). The PAI-2–AP chimeric protein was cloned in pBAD18-Kn, an inducible expression vector containing the promoter of the *araBAD* operon (Guzman *et al.*, 1995). This chimeric protein was exported inefficiently (D.Belin, S.Bost, L.M.Guzman and J.Beckwith, in preparation). Since a positive charge at the N-terminus of signal sequences facilitates protein export in *E.coli* (Iino *et al.*, 1987; Lehnhardt *et al.*, 1988), we substituted the two Glu residues at the PAI-2 N-terminus with Lys residues. Although these substitutions did not increase the export efficiency of the chimeric protein, the presence of the Lys AAA codon at position +4 to +6 led to a 6–10-fold increase in the synthesis of the chimeric protein. The high level expression of this PAI-2–AP chimeric protein was toxic: it prevented colony formation

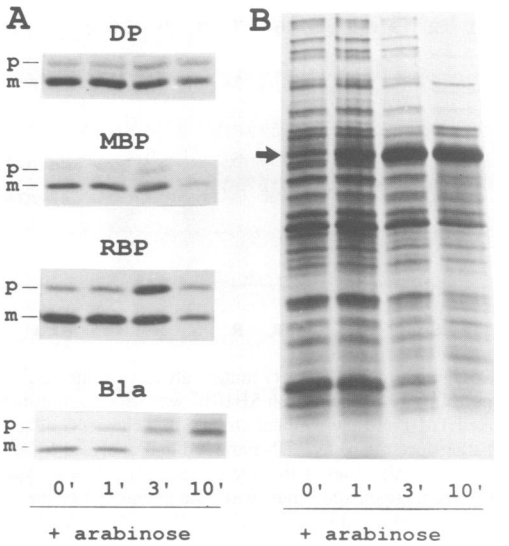

Fig. 1. The induction of the PAI-2–AP chimeric protein interferes with protein export and overall protein synthesis. (**A**) Cells were pulse-labelled for 15 s at 37°C at the indicated times after induction with arabinose. Lysates were immunoprecipitated with anti-MBP/DP and anti-RBP antibodies or anti-Bla and anti-AP antibodies. The positions of precursor (p) and mature (m) proteins are indicated. The non-specific bands appearing after 3 min of arabinose induction in the Bla panel represent degradation products of the overproduced PAI-2–AP chimeric protein (not shown). Total cell lysates are shown in (**B**). The position of the PAI-2–AP chimeric protein is indicated by an arrow.

in the presence of arabinose, and induction with arabinose in liquid cultures led to a rapid and irreversible growth arrest. To determine whether this toxicity was caused by an interference of the chimeric protein with the export of wild-type proteins, cells were pulse-labelled at various times after the addition of arabinose, and the ratios of precursors to mature proteins for DegP (DP), maltose-binding protein (MBP), ribose-binding protein (RBP) and β-lactamase (Bla) were determined (Figure 1A). After a 3 min induction, the synthetic rate of the fusion protein was maximal (Guzman *et al.*, 1995) and an increased accumulation of the precursors was visible for all four proteins analysed. This secretion defect was more dramatic at later times and was accompanied by a decrease in the overall rate of protein synthesis (Figure 1B). These results suggest that the overexpression of the chimeric protein fusion 'jams' the *E.coli* translocation machinery, which, in turn, leads to an interference with protein export and growth arrest.

Suppressors of the PAI-2–AP toxicity map to sec genes

The parental strain which expresses the chimeric PAI-2–AP protein is unable to form colonies on plates containing arabinose. We isolated a large number of spontaneous and UV-induced suppressors of this Ara^S phenotype, which restore growth under inducing conditions whilst still allowing export of the chimeric protein. We found that 17 suppressors were linked to *secA*, 14 to *secY* and one to *secE*, whilst nine were not linked to any of the known *sec* loci tested. These mutations mapped to six different loci scattered around the chromosome. One of these loci was localized at 69 min on the *E.coli* chromosome. Since *secG* was recently mapped to this region (Nishiyama *et al.*,

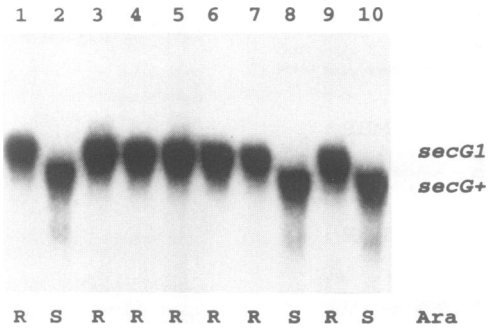

Fig. 2. Cosegregation of the *secG* mutant allele with the Ara^R phenotype. A P1 lysate grown on SB1010 was used to transduce the *secG*-linked Tn10 into the parental strain SB0. The *secG* alleles of the resulting transductants were PCR-amplified and analysed by SSCP. The positions of migration of the mutant (*secG1*) and wild-type (*secG*$^+$) DNA fragments are indicated. The phenotype of the transductants is indicated below each lane R, Ara^R; S, Ara^S.

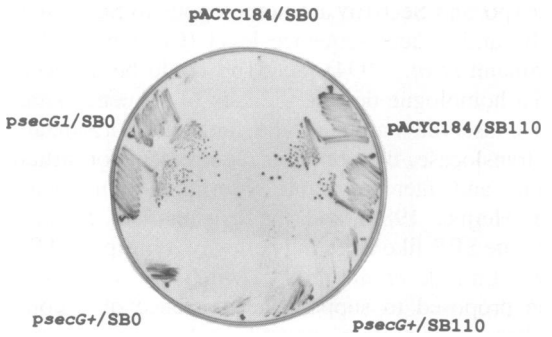

Fig. 3. The *secG1* allele is responsible for the Ara^R phenotype. The parental strain SB0 and the *secG* mutant strain SB110, which both carry the plasmid pBAD72K encoding the PAI-2–AP chimeric protein, were transformed with the empty pACYC184 vector, p*secG*$^+$:pACYC184 containing the *secG*$^+$ allele, or *psecG1*:pACYC184 containing the mutant *secG1* allele. Two independent colonies of each transformation were streaked on NZ plates supplemented with kanamycin, chloramphenicol, XP and 0.2% arabinose, and incubated for 20 h at 37°C.

1993), we PCR-amplified and sequenced the gene from this suppressor. One mutation was found in the *secG* coding sequence causing a Phe to Ser substitution at position 43 of SecG.

A suppressor mutation in secG

The *secG* mutation in SB110 could be responsible for the Ara^R phenotype or, alternatively, it could represent a silent polymorphic allele. The suppressor mutation in SB110 was linked to the Tn10 of CAG12072 with a frequency of 85%, and P1 lysates grown on Ara^R Tet^R transductants were able to cotransduce the suppressor phenotype into the parental strain at the expected frequency of 85%. The mutant and wild-type *secG* alleles can be distinguished by SSCP (Figure 2), which can detect single base substitutions according to the pattern of migration of single-strand DNA molecules in non-denaturing conditions. The mutant *secG* allele and the suppressor segregated together in 39/50 Tet^R transductants, whilst the remaining 11 Ara^S transductants retained the wild-type *secG* allele. We then subcloned both the wild-type and mutant *secG* in pACYC184, a plasmid compatible with pBAD18-Kn (see Materials and methods and Table I). Introduction of p*secG*$^+$ in SB110, the Ara^R strain which carries the mutant *secG* allele on the chromosome, restored the Ara^S phenotype, whilst p*secG1* was able to confer an Ara^R phenotype to DHB3, the *secG*$^+$ parental strain (Figure 3). Taken together, these results indicate that the *secG1* mutation is responsible for the Ara^R phenotype. Although this mutation is recessive to *secG*$^+$ expressed from a multicopy plasmid, it is dominant to *secG*$^+$ when expressed from a multicopy plasmid.

secG suppressors identify essential residues in the protein

The observed multicopy dominance of *secG1* allowed us to isolate additional *secG* suppressors. The *secG*$^+$ gene was subjected to PCR-mutagenesis and cloned into pACYC184. Upon transformation of the parental strain, many Ara^R suppressors could be isolated on rich plates supplemented with arabinose, and the *secG* inserts of plasmid-borne suppressors were sequenced. Among 28 *secG* suppressors, we found 15 single mutations, three double mutations and 10 suppressors with multiple mutations (ranging from three to eight substitutions), all localized to the coding sequence. Remarkably, all single mutations mapped to three contiguous codons coding for amino acids 41–43 in the second potential transmembrane domain of the protein (Figure 4). Two of the three double mutations and all 10 multiple mutations also had a mutation in one of these three contiguous codons. The only exception was a double mutant (*secG2*) which had two mutations in the third potential transmembrane domain. The most frequent mutation recovered encodes the F43S substitution, which is identical to the *secG1* mutation described above; this mutation was also recovered after mutagenesis of *psecG*$^+$ in a mutator *mutD* strain. Surprisingly, one of the mutations resulted in a very conservative Phe to Tyr substitution at position 43. These results suggest the existence of a critical site in the second potential transmembrane domain of SecG.

secG suppressors are phenotypically Sec at 37°C

Two types of mutations have been isolated in *sec* genes: *sec* mutations that decrease the export of wild-type proteins and *prl* mutations that improve the export of defective signal sequences; some *prl* mutations have also been shown to slow down the export of wild-type proteins (Stader *et al.*, 1989). To characterize further the *secG* suppressors, we first investigated their effect on the export of wild-type proteins. DB414, a derivative of Mph42 containing the pBAD24 plasmid, which carries the ampicillin resistance gene but does not express the PAI-2–AP fusion, was transformed with either *psecG*$^+$, *psecG1*, *psecG2* or pACYC184. The level of SecG in these strains was assayed by Western blot analysis (Figure 5). The steady-state level of SecG was slightly higher in strains carrying the *secG* plasmids than in a strain carrying the parental vector alone. In addition, SecG degradation products were detected in these strains, suggesting that a fraction of the overproduced SecG is unstable. Pulse-chase labelling experiments were performed at both 37°C and 23°C in these strains. At 37°C, strains expressing SecG1 or SecG2 accumulated the precursors of PhoA, OmpA, MBP and RBP in larger amounts than those found in the control strain (Figure 6). This increased level of

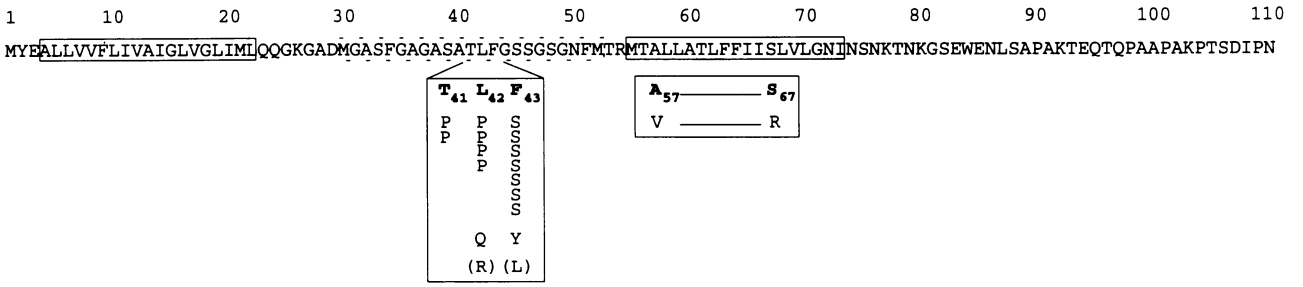

Fig. 4. Localization of the *secG* mutations conferring an Ara^R phenotype. The sequence of SecG (SWISSPROT AN: P33582) is represented in the one letter code. Potential transmembrane domains are indicated by solid and broken boxes. Wild-type SecG amino acids affected by the mutations are represented in bold characters below the sequence and the observed substitutions are aligned underneath. The two mutated amino acids linked by a line in the third transmembrane domain and the mutated amino acids shown in parenthesis are from double mutants. All the other substitutions are due to single mutations.

precursor proteins reflects a reduction in the kinetics of export, since the precursors were efficiently processed during a 2 min chase (data not shown). As shown in Figure 7, this effect was also evident at 23°C, where precursors accumulated to a much greater extent because of the intrinsic cold-sensivity of the export pathway (Pogliano and Beckwith, 1993). Thus, the *secG* suppressors are weak Sec mutants at both 37°C and 23°C. Surprisingly, in the strain carrying *psecG*$^+$, the amount of uncleaved precursors was reduced (Figure 6), suggesting that over-expression of SecG may accelerate protein export.

The Sec phenotype of the *secG* mutants was confirmed by using a collection of strains, carrying different signal sequence mutations in *malE*, that interfere to various extents with MBP export (Bedouelle *et al.*, 1980). The phenotype of these strains was scored on maltose indicator plates. All *secG* mutants decreased mutant MBP export, although this decrease was generally less severe with *secG2* than with the other mutants. Interestingly, all single mutations that altered codons 41– 43, including the Phe to Tyr substitution, affected each of the mutated *malE* signal sequences to the same extent, suggesting that these residues are all equally critical for SecG activity. MM4 has the most severe signal sequence defect and is able to grow on minimal maltose plates at 37°C but not at 23°C. Overexpression of the SecG mutant proteins in MM4 prevented growth on minimal maltose plates at 37°C, as shown in Figure 8 for *secG1*. The *secG2* mutation exerted a milder phenotype, since small colonies were detected after 2 days (not shown). In contrast, overexpression of wild-type SecG in MM4 accelerated growth on minimal maltose plates (Figure 8). Thus, overexpression of SecG$^+$ seems to facilitate the export mediated by both wild-type and mutant signal sequences at 37°C. This is reminiscent of the effect of SecD and SecF overexpression (Pogliano and Beckwith, 1994), although the mechanism by which these three proteins facilitate export may be different.

secG suppressors are not null mutants

Disruption of *secG* has been shown to decrease the kinetics of export of wild-type proteins at 23°C, but its effect at 37°C was only marginal (Nishiyama *et al.*, 1994). To determine whether the *secG1* mutation abolished all activity of SecG, we compared its phenotype with that of a disruption allele. The rate of signal sequence processing at 37°C in the different strains is shown in Figure 9. The amount of cytoplasmic precursors of several exported

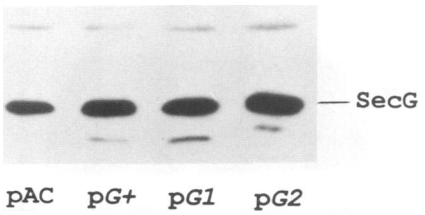

Fig. 5. Steady state level of SecG. Strain DB414 was transformed with the empty pACYC184 vector (pAC) or pACYC184 containing respectively *secG*$^+$ (p*G*$^+$), *secG1* (p*G1*) or *secG2* (p*G2*) (see Materials and methods). Equal amounts of total cellular proteins (10 μg) were analysed by immunoblotting with anti-SecG antibodies.

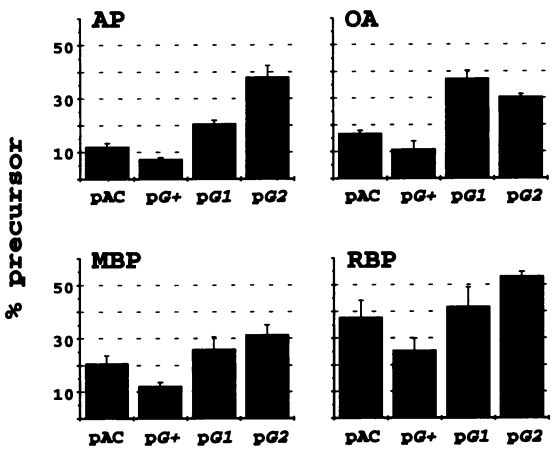

Fig. 6. Protein export at 37°C in *secG* mutant strains. The DB414 derived strains described in Figure 5 were grown at 37°C and pulse labelled for 15 s. Lysates were immunoprecipitated with anti-MBP and anti-RBP antibodies or with anti-AP and anti-OmpA antibodies. For each of the proteins analysed, the precursor and mature forms were measured, and the relative amount of precursor is indicated. Each bar graph represents the average from three independent cultures. Error bars indicate the SD. The significance of the results was measured using a Mann–Whitney non-parametric test. *P*-values were 0.03 for AP and RBP (p*G*$^+$ versus pAC), for AP and OA (p*G1* versus p*G*$^+$; p*G2* versus p*G*$^+$) and for MBP (p*G1* versus p*G*$^+$). *P*-values were 0.08 for the other pairs.

proteins was clearly increased both in the *secG* null and *secG1* strains, although the *secG* null strain exhibited a slightly more pronounced Sec phenotype. In addition, the effects of *secG1* and of the null allele on MBP export in strains carrying *malE* signal sequence mutations were indistinguishable (not shown). This suggested that the Ara^R phenotype of *secG1* could simply result from the

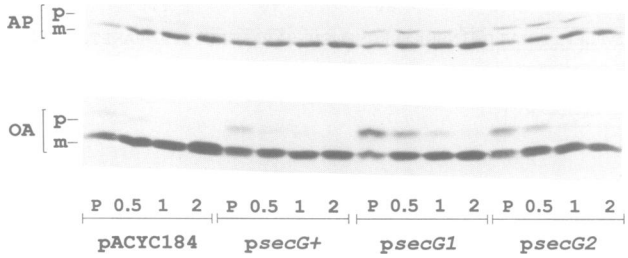

Fig. 7. Kinetics of AP and OmpA export at 23°C in *secG* mutant strains. The DB414 derived strains were grown at 37°C and shifted at 23°C for 3 h. Cells were pulse labelled for 20 s (P) and chased for the indicated length of time in min. Lysates were immunoprecipitated with anti-AP and anti-OmpA antibodies.

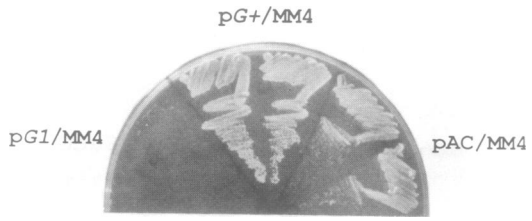

Fig. 8. Effect of wild-type and mutant *secG* alleles on MM4 growth. MM4 was transformed with empty pACYC184 vector (pAC), or pACYC184 containing $secG^+$ (pG^+) or secG1 (p*G1*). Two independent colonies of each transformation were streaked on minimal maltose plates supplemented with chloramphenicol and incubated for 36 h at 37°C.

synthesis of an inactive SecG protein. However, at 23°C, where the Sec defect of both strains was more severe, the *secG* null allele affected protein export to a greater extent than *secG1* (data not shown), suggesting that SecG1 retained some activity. Moreover, if *secG1* encoded a totally inactive protein, it would be expected that a *secG* disruption allele should abolish the toxicity of the PAI-2–AP chimeric protein. However, transduction of the *secG*::Kn disruption allele into a strain expressing the PAI-2–AP chimeric protein did not restore growth in the presence of arabinose. In liquid cultures, the $secG^+$ and *secG*::Kn strains both showed a rapid growth arrest after arabinose induction whereas the *secG1* strain continued to grow (Figure 10A). Thus, whilst both *secG* mutations decrease the kinetics of export of wild-type proteins, the *secG1* allele selectively prevents the toxicity of the chimeric PAI-2–AP protein. In an attempt to elucidate this selective effect of the *secG1* mutation, we analysed the kinetics of PAI-2–AP export upon induction with arabinose (Figure 10B). The maximal rate of PAI-2–AP synthesis is already achieved after 3–5 min of induction (Figure 1B; Guzman *et al.*, 1995). The kinetics of PAI-2–AP export were indistinguishable in $secG^+$ or *secG*::Kn strains. In contrast, the accumulation of active enzyme was markedly slower in the *secG1* strain. This effect of the mutant SecG1 protein was specific for the chimeric PAI-2–AP protein, since the kinetics of export of wild-type alkaline phosphatase expressed from the same vector were indistinguishable in the *secG1* or $secG^+$ strains (Figure 10C). Thus, the null allele is clearly different from the Ara^R suppressors that map in *secG*.

Finally, we tested whether the *secG* suppressors displayed the Cs phenotype of the *secG* disruption allele observed in C600 and W3110 backgrounds. Although

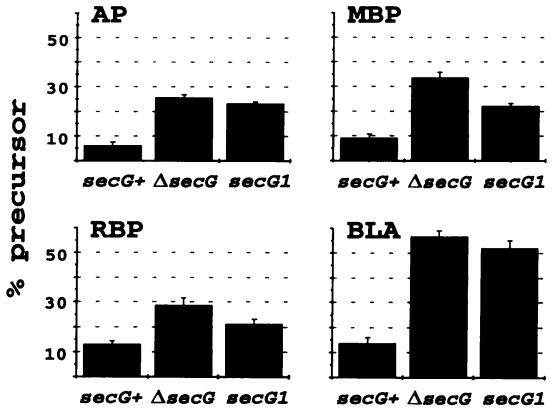

Fig. 9. Effect of *secG1* and of a *secG* null allele on protein export at 37°C. $secG^+$: wild-type chromosomic *secG* allele (DB414); Δ*secG*: disrupted *secG* allele (SB51); *secG1*: mutant *secG1* chromosomic allele (SB52). Cells were pulse-labelled for 15 s at 37°C. Lysates were immunoprecipitated with anti-MBP and anti-RBP antibodies or with anti-AP and anti-Bla antibodies. For each of the proteins analysed, the precursor and mature forms were measured, and the relative amount of precursor is indicated. Each bar graph represents the average from three independent cultures. Error bars indicate the SD.

neither the *secG* null allele nor the *secG* suppressor mutants resulted in a Cs phenotype in strains derived from MC1000 or MC4100, the chromosomally-encoded *secG1* mutation and all the plasmid-borne *secG* mutants were able to restore normal growth at 23°C in strains derived from KN370. Taken together, these results indicate that whilst the *secG* suppressors have an export defect, they do not simply encode an inactive protein.

Suppressors of secG mutants map to secY

As shown above (Figure 8), overexpression of SecG1 in MM4 prevented growth on minimal maltose at 37°C; this Mal^- phenotype was also conferred by the *secG* disruption allele. This phenotype facilitated the isolation of extragenic suppressors of *secG1* by selecting for Mal^+ revertants of strain SB75. Twenty-one suppressors were linked to *secY*, whilst twenty-five were linked to *malE*. Sixteen of the *secY*-linked suppressors were transduced into either MM4, SB70 or SB76. All these suppressors resulted in a Prl phenotype in MM4, which carries a chromosomal $secG^+$ gene, as determined on McConkey indicator plates. Nine suppressors also exhibited a Prl phenotype in SB70, which carries the *secG* disruption allele. Thus, whilst SecG activity is required both *in vivo* (at least at 23°C) and *in vitro* over a wide range of temperatures for an optimal protein export, translocons containing a mutant SecY can exhibit a Prl phenotype even in the absence of SecG. Seven of the *prlA* suppressors were unable to confer growth on minimal maltose in SB70, indicating that they required some SecG activity to allow export mediated by the mutant signal sequence; five of those were as efficient in SB75 as in SB76. In these mutants, the residual SecG activity can be provided by proteins mutated in either the second or the third potential transmembrane domain. However, two *prlA* alleles were unable to confer a Mal^+ phenotype when *secG1* was replaced by the *secG2* allele. This suggests that these two *prlA* alleles have a specific requirement for the wild-type SecG third potential transmembrane domain.

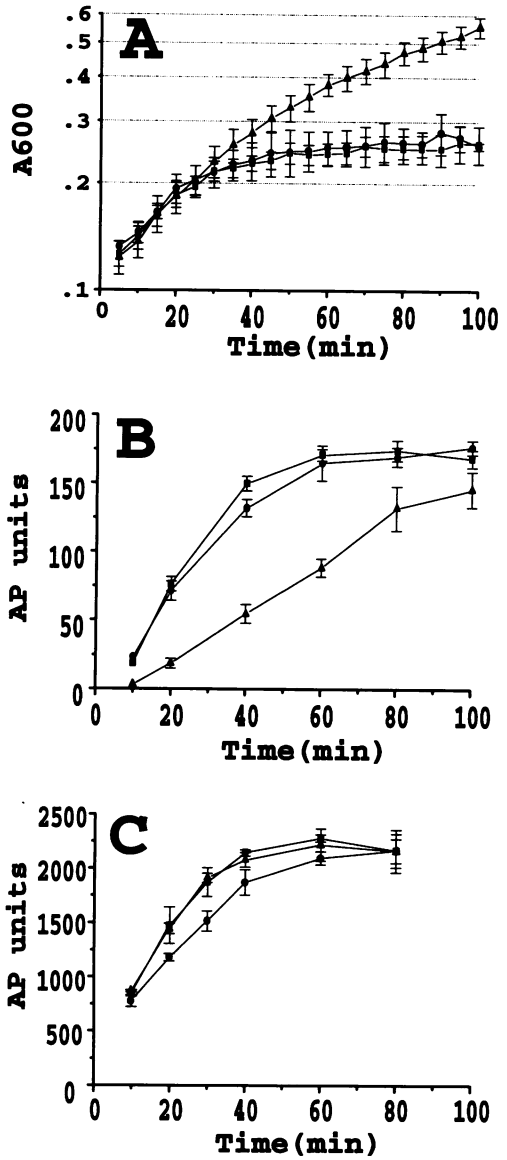

Fig. 10. Effect of *secG1* and of a *secG* null allele on growth and on the kinetics of PAI-2–AP and wild-type PhoA export. Cells were grown to an A_{600} of 0.1 in NZ medium at 37°C and induced with 0.2% arabinose at time 0. Samples were taken every 5 min to measure the A_{600} of the cultures; AP activity was assayed at the indicated times. (■) = $secG^+$ strain, (▲) = *secG1* strain and (●) = *secG::Kn* strain. Each curve represents the average from three independent cultures. Error bars indicate the SD. (**A** and **B**) The strains carry the pBAD72K plasmid and express the chimeric PAI-2–AP protein. (**C**) The strains carry the pDB3 plasmid (Guzman *et al.*, 1995) and express the wild-type PhoA protein.

Discussion

The signal sequence of murine PAI-2 can promote the export of alkaline phosphatase into the periplasm of *E.coli*. However, this export is inefficient, a situation similar to that which occurs in the ER of mammalian cells which produce PAI-2 (Belin *et al.*, 1989). High level expression of the PAI-2–AP chimeric protein led to a rapid and irreversible growth arrest. This effect is due to the functional activity of the mPAI-2 signal sequence, since overexpression of the mature portion of AP, which is not exported, is not toxic. A reduced rate of protein synthesis, such as that mediated by sublethal concentrations of antibiotics, can suppress several mutants defective in protein secretion (Lee and Beckwith, 1986). However, the toxicity of the chimeric protein was not suppressed by sublethal concentrations of chloramphenicol or tetracycline (data not shown). We have therefore taken advantage of the toxicity of the chimeric protein, which is expressed under the control of the arabinose p*BAD* promoter and hence confers an Ara^S phenotype, to develop a new genetic selection for the study of the components of the *E.coli* translocation machinery. Most of the Ara^R suppressors mapped to known *sec* genes, indicating that the irreversible growth arrest is caused by an altered interaction of the chimeric protein with the export machinery. This validates the use of our selection for the genetic characterization of the components of the translocation machinery.

In contrast to previous genetic selections, this study was based on the use of an inefficient signal sequence fused to the mature portion of a periplasmic protein. This selection could have been expected to yield *sec* mutations. Such mutants would be unable to export the fusion protein and are not easily distinguished from the large spectrum of mutations which directly or indirectly prevent the expression of the chimeric protein. We therefore decided to concentrate our efforts on suppressors which still allow export of the chimeric protein. Such suppressors could facilitate the secretion of the chimeric protein by compensating for the inefficient signal sequence of mPAI-2. If this had been the case, our selection would have simply yielded a spectrum of *prl* alleles. However, only one of our suppressors, a *secY* allele, was able to export an alkaline phosphatase which lacked a signal sequence, and is therefore a strong *prlA* allele (Derman *et al.*, 1993); in addition, two strong *prlA* alleles, *prlA4* and *prlA LS-11*, did not answer the selection. Thus, our selection was principally directed toward the isolation of a class of weak *sec* mutants.

This selection enabled us to isolate the first *secG* point mutant, *secG1*. SecG has been proposed to act after SecA-mediated targeting to the SecY/E/G complex (Hanada *et al.*, 1994). This suggested that our selection was directed neither towards the targeting step of protein secretion, nor towards the final step of protein release into the periplasm, but was restricted to an early step of the translocation process (Pogliano and Beckwith, 1993). In this context, it seems interesting to note that the dissection of PAI-2 translocation in a mammalian *in vitro* system provided evidence that only a fraction of the nascent chains targeted to the translocation machinery actually cross the ER membrane, suggesting that signal sequences are also recognized at a second step, and that this recognition can contribute significantly to the overall efficiency of translocation (D.Belin, S.Bost, J.-D.Vassalli and K.Strub, in preparation).

It has been suggested that all the essential components of the general protein export machinery have been identified genetically. However, other genes could have escaped previous detection because mutations in these genes only affect a fraction of all exported proteins. For instance, *ffh*, the 4.5S RNA (Phillips and Silhavy, 1992; Miller *et al.*, 1994) and *ftsY* (Luirink *et al.*, 1994), which have been implicated in an alternative targeting pathway, were not identified in selections for secretion mutants. Similarly, *ftsH*, which encodes a putative membrane-bound ATPase

and was recently shown to influence protein translocation (Tomoyasu *et al.*, 1993; Akiyama *et al.*, 1994), was not identified by previous genetic selections. The clear bias of our selection toward suppressors with a weak Sec phenotype and the predominance of mutations in genes encoding the components of the translocase might have been both critical to the isolation of the *secG1* mutant. Whilst a *secG* deletion allele confers a severe secretion defect at 23°C, its Cs phenotype is only manifested in certain genetic backgrounds. However, all the *secG* point mutants described here were found to strengthen the Mal$^-$ phenotypes caused by several *malE* signal sequence mutations. Thus, *secG* mutations should appear in genetic screenings for unlinked mutations which decrease export mediated by mutant or inefficient signal sequences. The previous failure to isolate *secG* mutants in selections based on signal sequence–LacZ fusion proteins (Oliver and Beckwith, 1981; Kumamoto and Beckwith, 1983; Gardel *et al.*, 1987) or on *secA* regulation (Riggs *et al.*, 1988; Schatz *et al.*, 1989) can be attributed either to the small size of *secG* or to the fact that the *secG* mutants which have either null or weak Sec phenotypes do not answer these selections. Indeed, both *secG1* and a disruption allele (Nishiyama *et al.*, 1994) have a small effect on SecA expression.

Two possible topologies have been proposed for SecG, with either two or three membrane spanning segments; in both models, the C-terminal portion of the protein faces the cytoplasm. Remarkably, all *secG* single mutations isolated here map to three contiguous codons encoding amino acids 41–43. This portion of SecG is either periplasmic or embedded in the membrane. Mutations in codons 41–43 were responsible for both the specific effect on the kinetics of export of the chimeric protein and the general Sec effect on wild-type proteins and mutant MBP proteins. This suggests that amino acids 41–43 are essential constituents of the SecG active site. This notion is reinforced by the observation that the conservative F43Y showed the same export defect as less conservative substitutions which, for instance, introduce proline residues. However, amino acids 41–43 are probably not the only determinants of SecG function since *secG2*, a double mutant with two mutations in the third potential transmembrane domain of the protein, also caused a weak protein export defect. In an attempt to define other essential residues in SecG, multicopy dominant *sec* alleles of *secG* were isolated by screening for a Mal$^-$ phenotype in strain MM5. All these SecG mutants must be able to assemble with SecY/E and displace, at least to some extent, the normal protein encoded by the chromosomal *secG*$^+$ allele. Interestingly, eight out of 10 mutants isolated mapped to the second half of the protein, beyond position 70 of SecG (Figure 4). Two of these mutants encode fusion proteins that lack three or 33 C-terminal residues of SecG, and two others were single point mutations (N74D and Q94R), which suggests that this region is also necessary for SecG function. When these *secG* mutants were expressed in the MM1–5 strains, their phenotypes were weaker than that of *secG1* and similar to that conferred by *secG2*. The two remaining mutants were a S39A and a G44S substitution. Surprisingly, none of these mutations did suppress the toxicity of the chimeric mPAI-2–AP protein. Since two mutations flanking the $T_{41}L_{42}F_{43}$ site do not answer our selection, one site of SecG required for the toxicity of the chimeric protein appears restricted to these three amino acids. Taken together, these data indicate that the normal activity of SecG involves at least three regions: the central TLF domain, the third transmembrane domain and the C-terminal tail. This cooperation of protein domains is reminiscent of recent results obtained with SecE: the second cytoplasmic domain is indispensable for SecE activity, whereas the third membrane spanning segment appears to be only required to maintain the proper topological arrangement of the cytoplasmic domain (Murphy and Beckwith, 1994). Analysis of the database for *secG* homologues has only detected a *Mycobacterium leprae* ORF which is tentatively called *secG*_MYCLE. This protein has a 22% identity with its *E.coli* homologue and is predicted to contain two potential transmembrane domains. Interestingly, the TLF domain of the *E.coli* protein defined here is also present in SecG_MYCLE.

SecG is a component of the translocase which interacts with SecY/E and contributes to the rapid export of secreted proteins (Hanada *et al.*, 1994; Nishiyama *et al.*, 1994). A *secE* mutation which reduces the expression level of SecE specifically reduced the level of SecG and, to a minor extent, that of SecY (Nishiyama *et al.*, 1993). Although these results suggest an interaction between these three subunits, the function of SecG is far from being completely understood. A subunit dynamics study in the preprotein translocase showed that SecY and SecE remain stably associated *in vivo*, whereas SecG can be exchanged between SecY/E complexes (Joly *et al.*, 1994). Some of the SecG mutants described here could affect the exchange of SecG. A recycling of SecG is compatible with the existence of two types of SecY/E complexes. SecG-containing complexes would be fully active, whilst complexes lacking SecG may be less so. This possibility is consistent with the observation that overexpression of SecG accelerates wild-type protein export by titrating the postulated SecY/E complexes to provide fully active complexes.

In conclusion, the selection described here allows the detection of subtle secretion defects which prevent jamming of the secretion machinery by a secretable protein whilst still allowing overall protein export. The isolation of several *secG* mutations, and the different phenotypes conferred by these mutations and by a disruption allele suggest that SecG contributes to the functional activity of signal sequences and may therefore play a more dynamic role in protein export than previously anticipated.

Material and methods

Reagents

Liquid and solid media were prepared as described (Miller, 1992). Antibiotics were used at the following concentrations: kanamycin 40 μg/ml, ampicillin 200 μg/ml, chloramphenicol 30 μg/ml and tetracycline 15 μg/ml. 5-bromo-4-chloro-3 indolyl phosphate (XP) was purchased from DCL (Oxford, CT) and used at a concentration of 40 μg/ml. The rabbit anti-SecG antibody, which was raised against the synthetic peptide (CQTQPAAPAKPTSDIPN), was a kind gift from Dr H.Tokuda. Rabbit antisera against MBP, OmpA and RBP were kind gifts from Dr J.Beckwith. Rabbit anti-bacterial alkaline phosphatase (7.2 mg/ml) and rabbit anti-bacterial β-lactamase (18.7 mg/ml) were purchased from 5prime–3prime, Inc. (Boulder, CO).

Bacterial strains

All strains are described in Table I and were constructed by P1-mediated transduction or by transformation (Miller, 1992).

Table I. *Escherichia coli* strains

Strain	Genotype	Reference
MC1000	F⁻ *araD*139 Δ(*ara–leu*)$_{7696}$ Δ(*lac*)X74 *rpsL*150 *galU galK thi*	Casadaban and Cohen (1980)
DHB3	MC1000 *malF*Δ3 *PhoA*Δ(*Pvu*II) *phoR*	Boyd *et al.* (1987)
DHB4	DHB3/ F′*lacI*^{Q} *pro*	Boyd *et al.* (1987)
Mph42	MC1000 *phoR*	Beckwith collection
DB414	Mph42 pBAD24	this study
MC4100	F⁻ *araD*139 *relA*1 *thi rpsL*150 *flbB*5301 Δ(*argF–lac*) U169 *deoC7 ptsF*25 *rbs*R	Casadaban (1976)
KN370	C600 *recD*1009 Δ*secG*::Kn	Nishiyama *et al.* (1994)
SB0	DHB3 pBAD72K	this study
SB110	DHB4 *secG1* pBAD72K	F43S mutant
SB1010	SB0 *secG1 zgj*-203::Tn*10*	this study
SB50	SB0 Δ*secG*::Kn	this study
SB51	DB414 Δ*secG*::Kn	this study
SB52	DB414 *secG1 zgj*-203::Tn*10*	this study
SB53	DB414 p*secG1*	F43S mutant, in pACYC184
SB54	DB414 p*secG2*	(A57V, S67R) mutant, in pACYC184
SB60	C600 *recD*1009 secG1 *zgj*-203::Tn*10*	this study
SB61	KN370 p*secG1*	this study
SB62	KN370 p*secG2*	this study
MM60	MC4100 *leu*::Tn*10*	Beckwith collection
CG107	F⁻*secD*$^{+}$::Tn*10* (*ara–leu*)$_{1119}$ *trp mal supF relA rpsL thi*	Gardel *et al.* (1987)
PS142	MC4100 *argE*::Tn*10 secA*::*lac*f181 (λPR9 *secA*$^{+}$)	Beckwith collection
KJ195	MC4100 *secY*$^{+}$::Tn*10 secA*::*lac*f181 (λPR9 *secA*$^{+}$)	Beckwith collection
CAG12072	MG1655 *zgj-203*::Tn*10*	Singer *et al.* (1989)
MM1	MC4100 *malE*10-1	Bedouelle *et al.* (1980)
MM2	MC4100 *malE*14-1	Bedouelle *et al.* (1980)
MM3	MC4100 *malE*16-1	Bedouelle *et al.* (1980)
MM4	MC4100 *malE*18-1	Bedouelle *et al.* (1980)
MM5	MC4100 *malE*19-1	Bedouelle *et al.* (1980)
SB70	MM4 Δ*secG*::Kn	this study
SB71	SB70 p*secG1*	this study
SB72	SB70 p*secG2*	this study
SB73	SB70 pACYC184	this study
SB74	SB70 p*secG*$^{+}$	*secG*$^{+}$, in pACYC184
SB75	MM4 p*secG1*	this study
SB76	MM4 p*secG2*	this study

Plasmid construction

pBAD72K was constructed as follows: a *Kpn*I–*Xba*I fragment from pSWFII (Ehrman *et al.*, 1990) was subcloned into the cognate sites of pBAD18 (Guzman *et al.*, 1995). pBAD18 contains the promoter of the arabinose operon upstream of a multicloning site, the gene encoding the regulator AraC and an ampicillin resistance cassette; this yielded pBAD18/Tn*phoA*. The sequence coding for the amino acids 1–52 of mPAI-2 was PCR-amplified with primers P2D (5′CCTAGCTAGCAGG-AGGAATTCACCATGPuAAPuAAC TTTCC3′) and P2F (5′GGGGTA-CCTGTTCAGTGTTACC3′), restricted with *Nhe*I and *Kpn*I and subcloned into the cognate sites of pBAD18/Tn*phoA*. This introduces a Shine–Dalgarno sequence and an ATG downstream of the *Nhe*I site. The positions corresponding to nucleic acid +4 and +7 of mPAI-2 are degenerate in P2D. This allowed the substitution of one or two Lys residues for the two wild-type Glu residues. The N-terminal sequence of the fusion protein encoded by pBAD72K is Met–Lys–Lys. Finally, a *Sal*I fragment from pUC4Kn (Pharmacia), encoding a kanamycin resistance gene, was subcloned downstream of the TnPhoA fragment. pBAD24 is a derivative of pBAD18 (Guzman *et al.*, 1995).

psecG^{+} was constructed as follows: the *secG* promoter and coding sequence were PCR amplified from chromosomic DNA (Russo *et al.*, 1993) with primers P12Up (5′CAATGCGATAAAATTGCCC3′) and P12Don (5′TACCAATTCCACCACCTCG3′). The 500 bp fragment was subcloned into the *Sma*I site of pUC19. The *Eco*RI/Klenow–*Hin*dIII fragment of pUC19*secG*$^{+}$ was subcloned between the *Hin*dIII and *Eco*RV sites of pACYC184. p*secG1* and p*secG2* are derivatives of p*secG*$^{+}$ which have the F43S and (A57V, S67R) mutations, respectively.

Isolation of suppressors of PAI-2–AP toxicity

Spontaneous mutants of SB0 were isolated by spreading 10^{8} cells from independent cultures onto NZ plates supplemented with 0.2% arabinose and 40 μg/ml of XP. After 24 h at 37°C and 2–3 days at room temperature, suppressors appeared with a frequency of 10^{-7}. Blue colonies (30% of all colonies) were purified on selective plates at 37°C; only one colony was kept for each plate to ensure the isolation of independent suppressors. UV suppressors were isolated after irradiation for 45 s to 1 min, a dose which causes a 99% lethality (Miller, 1992). The UV-induced suppressors appeared with a frequency of 10^{-6}, and on the average one day earlier than the spontaneous mutants. Eighteen of the 24 spontaneous suppressors were due to mutant plasmids. Among the 91 UV induced suppressors, 27 were plasmid-borne and 64 chromosomic. *pcnB* mutants, identified by a low plasmid copy number (n = 18), and mutants which had an AP activity lower than that of the parent strain (n = 5) were eliminated. The 41 remaining Ara^{R} suppressors were first mapped by transduction with a Tn10 transposon linked to either *secA*, *secY*, *secE* or *secD/F*. All the suppressors analysed were linked to a Tn10 and transduced in the parental background. Thirty-six out of 40 were able to confer an Ara^{R} phenotype to SB0. All the *secA* mutations were dominant against a *secA*$^{+}$ allele on a F′ factor and recessive against a *secA*$^{+}$ allele expressed from the compatible vector pACYC184 (Kim *et al.*, 1994).

Isolation of Mal^{+} *suppressors of psecG1*

SB75 strain was unable to grow on minimal maltose at 37°C. UV-mutagenesis was performed as described above, and 0.3 units of A_{600} of irradiated cells were plated on minimal maltose plates containing chloramphenicol. The plates were incubated at 37°C for two days. The Prl phenotype of the suppressors was scored on McConkey plates supplemented with 1% maltose.

Alkaline phosphatase assay

AP activity was measured by determining the rate of *p*-nitro-phenyl-phosphate (Sigma) hydrolysis in permeabilized cells, and was normalized to the A_{600} of the cell suspension (Michaelis *et al.*, 1983). The rate of chimeric PAI-2–AP protein synthesis was determined by 30 s pulse-labellings with [^{35}S]-methionine and immunoprecipitation, and it was normalized to that of OmpA or of Bla.

Pulse-labelling and immunoprecipitation

Cells were grown overnight in M63-glycerol medium supplemented with all 18 amino acids except methionine and cysteine, diluted 1:40 and

grown at 37°C to an A_{600} of 0.2. Cells were induced with 0.2% arabinose, 0.2% maltose or both for 1 h. Cells were labelled with 15 μCi/ml ^{35}S-methionine (Amersham SJ1015, 1000 Ci/mmole) for 15 s or 20 s as indicated. Labellings were terminated by transferring 0.7 ml of cells to prechilled Eppendorf tubes containing 0.7 ml of 0.2% methionine. Cells were centrifuged for 2 min at 13 000 r.p.m. at 4°C, resuspended in 50 μl of SDS buffer (1% SDS, 10 mM Tris–HCl pH 8, 1 mM EDTA) and heated for 2 min at 90°C. After 5 min at RT, 800 μl of KI buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 2% Triton-X-100) was added. After a 10 min incubation on ice, the lysates were centrifuged 10 min at 13 000 r.p.m. Antibodies were added to 300 μl of lysates. After 12 h at 0°C, immune complexes were bound to a 2–3-fold excess of fixed *StaphylococcusA* cells (IgGsorb) for 20 min on ice. The cells were washed twice with 1 ml of HB (50 mM Tris–HCl pH 8, 1 M NaCl, 1% Triton-X-100, 1 mM EDTA) and once with 1 ml of 10 mM Tris–HCl pH 8. Pellets were resuspended in 50 μl SB$^+$ (50 mM Tris–HCl pH 6.8, 5% β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue and 15% glycerol) and boiled for 2 min immediately before loading on 10% SDS–polyacrylamide gels. Gels were fixed, dried and exposed on XAR films or on phosphorscreen (Molecular Dynamics). Quantification was performed by scanning with a Molecular Dynamics phosphorimager and analysis with Image Quant version 3.22 software.

SSCP analysis

P12Up and P12Don primers were labelled with γ^{32}P-ATP (Amersham) and T4-polynucleotide kinase for 30 min at 37°C. Reactions were stopped by boiling 5 min at 95°C. The labelled and unlabelled primers were mixed 1:5 and used to amplify the chromosomic *secG* as described by Russo *et al.* (1993). The PCR products were boiled for 20 min before loading on Mutation Detection Enhancement Gels, prepared according to the manufacturer's instructions (AT Biochem, Inc.) and containing 10% glycerol. The gels were run at 4°C for 15 h at 40 W, dried and exposed to XAR films (Kodak).

PCR mutagenesis of secG

PCR mutagenesis of *secG* was performed using pUC19*secG*$^+$ and the reverse and forward primers of pUC19. The method described by Leung *et al.* (1989) yielded a very high rate of mutations (~4%) whereas the method described by Spee *et al.* (1993) provided a more acceptable mutation rate (<2%). We used a concentration of 20 μM of limiting dNTP and 200 μM of dITP. The mutated fragments were subcloned into pACYC184 as described above. DNA sequencing was performed using a Sequenase kit version 2.0 (USB) using 2 μl of plasmid DNA.

SDS–PAGE and immunoblotting

SDS–PAGE was performed according to Laemmli (1970), using slab gels with 10–15% separating gels and 4.5% stacking gels. The gels were soaked in transfer buffer (20 mM Tris–HCl pH 8.8, 0.1% SDS, 20% isopropanol). Proteins were transferred to 0.45 μm nitrocellulose membranes (Schleicher and Schuell) using a semi-dry transblot apparatus (Bio-Rad) for 1 h at 450 mA. The membranes were blocked for 1 h at 23°C in TBS-T (50 mM Tris–HCl pH 8, 150 mM NaCl, 0.1% Tween 20) containing 5% non fat dry milk, followed by a 1 h incubation with anti-SecG antibody diluted 1:1000 in TBS-T-milk. The membranes were washed twice for 15 min with TBS-T and incubated for 1 h at room temperature with TBS-T-milk containing horseradish peroxidase-coupled goat anti-rabbit antibody (Bio-Rad) diluted 1:1000. After three 20 min washes with TBS-T, bound antibodies were detected by the ECL system (Amersham) using XAR films (Kodak).

Acknowledgements

D.Belin thanks Jon Beckwith for his hospitality and for stimulating discussions on the design of genetic selections, Joe Pogliano for suggesting the use of the MM strains to characterize the *secG* mutants, Dana Boyd for computing the SecG topology models and all the members of the Beckwith lab, past and present, for their enthusiasm, criticism and imagination. We thank Dr H.Tokuda for strains and anti-SecG antibodies; Pierre Vassalli and Costa Georgopoulos for many helpful discussions and for a critical reading of the manuscript; Dr B.Conne for help with the SSCP experiments. S.Bost is a fellow of the MD/Ph.D. program and received a fellowship from the De Reuter Foundation. This work was supported by a grant from the Swiss National Science Foundation and by the Canton de Genève.

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Received on 29 May, 1995; revised on 10 July, 1995

Note added in proof

High level expression of the N-terminal 57 amino acids of mPAI-2 was also toxic, albeit not as much as that of the chimeric protein. The *secG1* allele also suppressed the toxic phenotype of this N-terminal fragment.